



## RESEARCH ARTICLE

### Bacteriological, Metabolic and Immunological Evaluation of European sea Bass Reared in Ponds with Heated Water under a Natural Vibriosis-Like Outbreak

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#### ABSTRACT

Fish culture in ponds with heated water offers better fish growth compared to farms in natural sea water temperature, but the increase in temperature favors the development of bacteria and fish disease. In this study, in European sea bass (*Dicentrarchus labrax*) specimens reared in ponds with heated water from a power plant showing symptoms of a vibriosis-like disease we aimed to characterize the bacterial community as well as some serum metabolic and immune parameters. Bacteriological analysis revealed an important contamination with fecal coliforms, *Pseudomonas aeruginosa*, *Vibrio alginolyticus*, yeasts and molds whilst fish tissues from both healthy and diseased specimens were positive for total aerobic flora and several potential pathogenic bacteria such as *V. alginolyticus*, *Aeromonas hydrophila*, *Pasteurella multocida*, *P. aeruginosa*, *Proteus vulgaris* and *P. mirabilis*. Additionally, *Grimontia hollisae* was only present in diseased fish tissues. Serum metabolic parameters such as aspartate aminotransferase, bile acids, creatine kinase, uric acid, glucose, globulin and potassium were generally increased in diseased fish though only the levels of phosphorous reached a significant level compared to the healthy specimens. Regarding fish innate immunity, our results showed no statistical differences between healthy and diseased fish on peroxidase, protease or antiprotease activity while the bactericidal activity against *V. anguillarum* was significantly decreased in diseased fish. Lastly, the gene expression of *illb*, *c3*, *dic* and *mpx* was down-regulated in the blood of diseased fish while *ighm* was up-regulated.

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#### INTRODUCTION

Aquaculture is nowadays one of the fastest growing food industries. However, this is still very limited in some areas such as the sub-Saharan Africa. In Algeria, it was virtually stagnant for many years, but had a relatively good production in 2008 and 2009. Its current trend is probably the development of marine fish farming structure, such as European sea bass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) (FAO, 2014). The most common fish production technologies in that country are cages in coastal areas and terrestrial ponds. For marine fish farming, at present, there are only two projects in Algeria, both dedicated to the two fish species previously mentioned. The first, located in

Azeffoune (TiziOuzou), is a floating aquaculture fish farm. The second one, aim of our study, is located in Cap Djinet (Boumerdes) and develops the fish breeding in terrestrial ponds with heated water coming from a power plant. However, the rapid development and growth of aquaculture has led to outbreaks of diseases in fish farms which cause severe economic loss, and constitute one of the main limiting factors.

Among the bacterial diseases affecting European sea bass, vibriosis at early life stages provokes the highest mortality. Vibriosis is one of the most prevalent bacterial fish diseases and is caused by several members of the genus *Vibrio* and related genera within the *Vibrionaceae* family (Austin and Austin, 2007). This bacterial family thrives in warm brackish and sea water and is widespread

in coastal marine and estuarine environments. As a result of global warming, *Vibrio* species have also spread to new temperate ecosystems (Baker-Austin *et al.*, 2012). *Vibrio* spp. are in many cases facultative pathogens that can readily colonize external lesions or, as part of the fish normal intestinal flora, set off an infection, presumably when a predisposing stress factor generates a failure in the mechanism regulating posterior gut permeability. Virtually all species of marine and estuarine fish of all ages are susceptible. *Vibrio* (*Listonella*) *anguillarum*, *V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus*, *V. harveyi* and *V. ordalii* have been associated with mortalities in farmed and feral fish in temperate and warm waters (Actis *et al.*, 2011). Identification of the species may present some difficulties as the taxonomy of the *Vibrionaceae* has undergone numerous recent revisions and is still changing.

Fish vibriosis is characterized by a systemic haemorrhagic septicemia and fish show signs as anaemia, lethargy, skin darkening, corneal thickening, erythema of the vent and the base of the fins, congested visceral blood vessel and fluid accumulation in the intestines (Toranzo *et al.*, 2005; Colorni and Diamant, 2014). In both aquaculture and larviculture, this disease is responsible for severe economic losses worldwide. This is especially significant for European sea bass, a species very sensitive to stressors and pathogens, where infections may occasionally cause significant economic losses. The aim of this work focused on European sea bass cultured in ponds using power plant heated water in Algeria under a natural outbreak resembling vibriosis. Thus, we evaluated the occurrence of bacterial pathogens in water and European sea bass specimens as well as the immune status.

## MATERIALS AND METHODS

**Fish farm and case presentation:** The ONDPA (Office National Aquaculture Development and Protection), located in the town of Djinet, Algeria, produces seabream and sea bass in ponds fed by heated sea water as a result of rejection of a nearby power plant. In September 2014, a clinical outbreak of disease with a high mortality was detected in several ponds of juvenile sea bass. The external clinical signs of the disease included fin erosion (especially caudal fin rot), circular ulcerative lesions and petechial and hemorrhagic skin in abdominal and thoracic region, fitting very well with a vibriosis-like disease (Toranzo *et al.*, 2005).

**Fish and water sampling:** One pond without apparent signs of disease served as control while other three ponds containing fish with clear signs of the disease were used to sample the diseased fish. Twenty juvenile of European sea bass specimens with a weight of  $52.6 \pm 9.4$  g was sampled (5 samples per pond) (15 diseased and 5 apparently healthy fish) in October 2014. Water temperature, pH and salinity were of 27°C, 8.28 and 36.5‰, respectively.

Blood samples were collected from the caudal vein. Fresh blood samples were immediately frozen for gene expression studies while others were allowed to clot at 4°C and centrifuged. The serum was frozen at -80°C until use for metabolic immunological analysis. Fragments from gills

and intestine were blended with 225 ml of buffered peptone water. This stock solution was used for the detection of different microorganisms. Water samples from each pond were also collected at a depth of 15 to 20 cm below the water surface using sterile 500 ml plastic bottles.

### Microbiological analysis of water and tissue samples:

Microbiological analyses were performed on freshly isolated water and tissue samples using common reagents (all from BioMérieux) and techniques (Eaton *et al.*, 1995) to detect and identify total and fecal coliforms, total aerobic flora, yeasts and molds, faecal enterococci, *Salmonella* spp., *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Vibrio* spp. or *Clostridium perfringens*. Isolated potential pathogens were also evaluated by using an API20E test.

### Determination of metabolic parameters in serum:

The activity of aspartate aminotransferase (AST) and creatine kinase (CK) as well the levels of uric acid (UA), glucose (GLU), calcium (CA<sup>++</sup>), phosphorus (PHOS), total protein (TP), albumin (ALB), globulin (GLOB) and potassium (K<sup>+</sup>) were determined in the serum of European sea bass specimens using samples of 100 µl of serum in an automated analyzer (VetScan, Abaxis Veterinary Diagnostics) and rotor (VetScan Aviane Reptilian Profile Plus) according to the manufacturer's instructions.

**Serum innate immune parameters:** The peroxidase (Quade and Roth, 1997), protease (Ross *et al.*, 2000), antiprotease (Hanif *et al.*, 2004) and bactericidal (Sunyer and Tort, 1995) activities were determined as elsewhere in serum samples.

### Gene expression by real-time PCR (qPCR):

Relative gene expression was analyzed in blood samples using real-time PCR (qPCR) and performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems) as previously described (Cerezuela *et al.*, 2013). These genes code for β subunit of T-cell receptor (*trb*), heavy chain of immunoglobulin M (*igmh*), eosinophil peroxidase-like (*mpx*), interleukin-1β (*il1b*), lysozyme (*lyz*), complement component 3 (*c3*), alkaline phosphatase (*alp*), dicentracin (*dic*) and hepcidin (*hamp*). For each mRNA, gene expression was corrected by the elongation factor 1-alpha (*ef1a*) content in each sample. The primers used are shown in Table 1.

**Statistical analysis:** Data were statistically analyzed by Levene's test for equality of variances and independent samples T-test for comparison of means (P<0.05).

## RESULTS

### Microbiological analysis revealed the presence of *Grimontia hollisae* only in diseased fish:

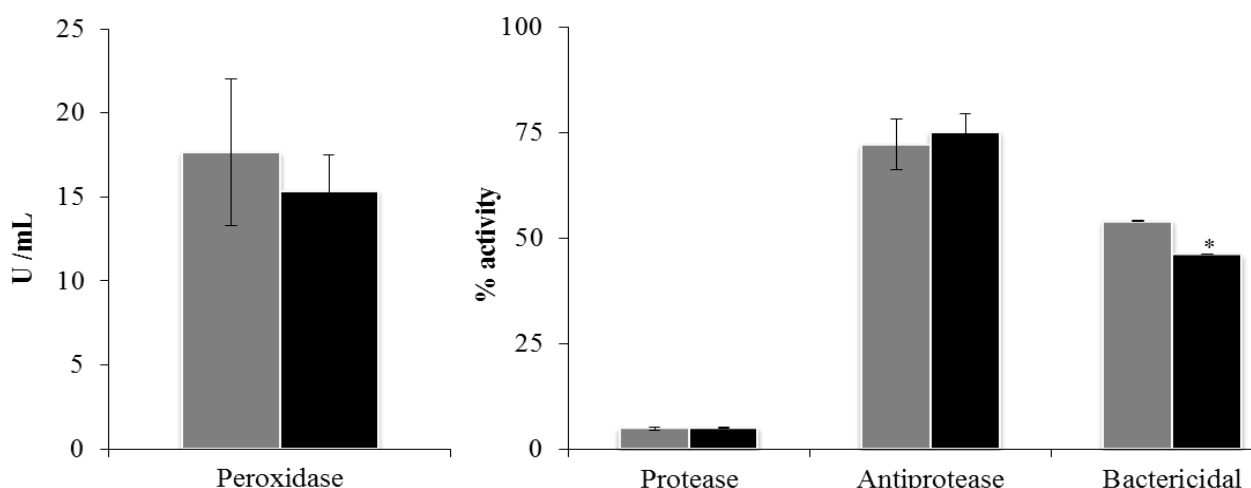
We performed a general bacteriological analysis in water and fish samples. The results of the present work revealed an important contamination of the water by different microorganisms such as fecal coliforms, *P. aeruginosa*, *V. alginolyticus*, yeasts and molds (Table 2). On the other hand, microbiological analysis of fish tissues was positive for

total aerobic flora and several potential pathogens (Table 3). The isolated bacteria were identified by the API20E kit and resulted to be *V. alginolyticus*, *Aeromonas hydrophila*, *Pasteurella multocida*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *P. mirabilis*. All the bacterial species isolated from diseased fish were also present in apparently healthy fish except *Grimontia hollisae*, which was only present in diseased fish tissues (Table 3).

**Metabolic parameters in serum from diseased sea bass specimens were generally increased:** Several metabolic parameters were determined in serum of apparently healthy and diseased sea bass specimens. In diseased fish the measured parameters were increased though only the levels of phosphorous reached a significant level (Table 4) due to the great fish-to-fish variability. For example, aspartate aminotransferase, bile acids, creatine kinase, uric acid, glucose, globulin and potassium levels were increased higher than two-fold in diseased fish compared

to the levels found in apparently healthy specimens but they never reached significance. However, phosphorous levels were increased in diseased fish 1.9-fold (from 3.87 to 7.37 mg/L) and this increment resulted significant. In the case of calcium, total protein and albumin there were very little increments in diseased fish specimens. Interestingly, the globulin/albumin ratio was increased from 0.52 to 0.85 in diseased fish.

**Immune response against bacterial infections was decreased in diseased fish:** Serum protease, antiprotease and peroxidase activities were not affected to a significant level by the bacterial infection (Fig. 1). By contrast, the serum bactericidal activity against *V. anguillarum* was significantly inhibited (Fig. 1). The expression of *il1b*, *mpx*, *c3* and *dic* was significantly lower in blood from diseased fish respect to the values detected in blood of healthy fish while the transcription of *ighm* was up-regulated in diseased fish specimens (Fig. 2).



**Fig. 1:** Peroxidase (U/mL), protease (%), antiprotease (%) and bactericidal (%) activities in serum of European sea bass apparently healthy (grey bars) or vibriosis-like diseased (black bars) specimens. Bars represent the means  $\pm$  SEM (n=5 healthy and 15 diseased specimens). Asterisk denotes significant differences between groups ( $P \leq 0.05$ ).

**Table 1:** Primers used for real-time PCR

Gene	Protein name	Account number	Sequence (5'-3')
<i>ef1a</i>	Elongation factor 1 $\alpha$	AJ866727	CGTTGGCTTCAACATCAAGA GAAGTTGCTGCTCCCTTGG
<i>il1b</i>	Interleukin 1 $\beta$	AJ269472	CAGGACTCCGGTTTGAACAT GTCCATTCAAAGGGGACAA
<i>lyz</i>	Lysozyme	FN667957	ATTTCTGGCTGGAACACAG GAGCTCTGGCAACAACATCA
<i>tcrb</i>	$\beta$ subunit of T-cell receptor	FN687461	GACGGACGAAGCTGCCCA TGGCAGCCTGTGTGATCTTCA
<i>ighm</i>	Heavy chain of immunoglobulin M	FN908858	AGGACAGGACTGCTGCTGTT CACCTGCTGTCTGCTTTGT
<i>c3</i>	Complement component 3	HM563078	ACCAAAGAAGCTGGCAACCAC CTAGCAGTCGGTCAGGGAAC
<i>alp</i>	Alkaline phosphatase	FJ860000	TTACCTCTGTGGGGTCAAGG TAGCCCATTTGAGGATGGAG
<i>dic</i>	Dicentracin	AY303949	GGCAAGTCCATCCACAAACT ATATTGCTCCGCTTGTGAT
<i>hamp</i>	Hepcidin	DQ131605	CCAGTCACTGAGGTGCAAGA GCTGTGACGCTTGTCTGT
<i>mpx</i>	Eosinophil peroxidase-like	DLAgn_00118340*	GAAGAGTGGGGCCTTTGTTT CTGGGCCCTCAGTGAAGACTC

\*Sequence obtained from <http://seabass.mpipz.mpg.de>.

**Table 2:** Microbiological analysis of water samples

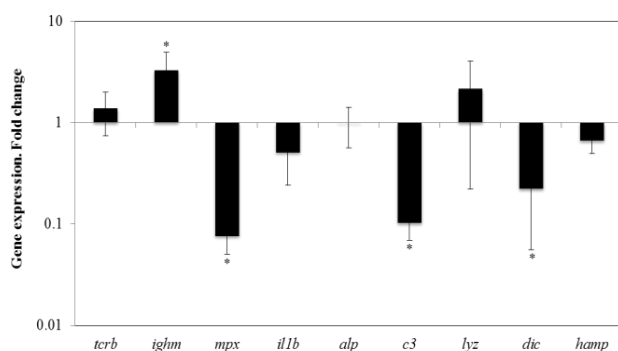
Total and fecal coliforms	Total aerobic flora	Yeast and molds	Fecal enterococci	<i>Salmonella</i> spp.	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>C. perfringens</i>	Potential pathogens
+	+	+	-	-	-	+	-	+ <i>V. alginolyticus</i>

**Table 3:** Microbiological analysis of fish tissues from healthy and diseased *D. labrax*

Group	Total aerobic flora	<i>Salmonella spp.</i>	<i>S. aureus</i>	<i>C. perfringens</i>	Potential pathogens
Healthy	+	-	-	-	<i>V. alginolyticus</i> , <i>Aeromonas hydrophila</i> , <i>Pasteurella multocida</i> , <i>Pseudomonas aeruginosa</i> , <i>Proteus vulgaris</i> , <i>P. mirabilis</i>
Diseased	+	-	-	-	<i>Grimontia (Vibrio) hollisae</i> , <i>V. alginolyticus</i> , <i>Aeromonas hydrophila</i> , <i>Pasteurella multocida</i> , <i>Pseudomonas aeruginosa</i> , <i>Proteus vulgaris</i> , <i>P. mirabilis</i>

**Table 4:** Metabolic parameters in serum of European sea bass healthy (n=5) or diseased (n=15) specimens. Data represent the mean±S.E.M. Data was analyzed by Levene's test for equality of variances and independent samples T-test for comparison of means. Asterisks denote significant differences between healthy and diseased specimens (P<0.05). Aspartate aminotransferase (AST), bile acids (BA), creatine kinase (CK), uric acid (UA), glucose (GLU), calcium (Ca<sup>++</sup>), phosphorus (PHOS), total protein (TP), albumin (ALB), globulin (GLOB) and potassium (K+)

Parameter	Units	Healthy	Diseased	Levene's test	P value
AST	U/L	13±1.73	26.83±6.68	0.018	0.202
BA	umol/L	9.00±0.82	22.33±4.11	0.203	0.145
CK	U/L	15.5±2.86	33.25±6.59	0.041	0.392
UA	mg/L	0.4±0.21	0.93±0.33	0.088	0.216
GLU	mg/L	93.10±55.52	217.67±52.09	0.431	0.308
CA	mg/L	2.13±0.03	2.35±0.26	0.049	0.586
PHOS	mg/L	3.87±0.15	7.37±1.06*	0.121	0.021
TP	g/L	1.83±0.38	2.33±0.20	0.687	0.33
ALB	g/L	0.83±0.15	1.03±0.16	0.222	0.387
GLOB	g/L	0.43±0.43	0.88±0.28	0.95	0.436
K+	mmol/L	0.33±0.33	0.73±0.50	0.177	0.526

**Fig. 2:** Gene expression determined by real-time PCR in blood of European sea bass vibriosis-like diseased specimens. The bars represent the mean±SEM (n=15 for vibriosis-like) fold change relative to control (healthy specimens; n=5). Asterisks denote significant differences between groups (P<0.05).

## DISCUSSION

European sea bass is a fish of great economic value in the Mediterranean area. Among the bacterial pathologies threatening this species, mainly when cultured at high densities in fish farms, vibriosis and pasteurellosis/photobacteriosis are among the most important and causes significant mortalities and economic losses (Balebona *et al.*, 1998; Romalde, 2002; Toranzo *et al.*, 2005; Essam *et al.*, 2016) and are caused by gram-negative bacteria in the family *Vibrionaceae*. Main identified causative bacterial agents have been identified as *V. anguillarum*, *V. alginolyticus*, *V. harveyi* or *P. damsela* spp. *piscicida* (Romalde, 2002; Pujalte *et al.*, 2003; Toranzo *et al.*, 2005; Austin and Austin, 2007; Damir *et al.*, 2013). These bacteria produce, amongst other lesions, skin ulcers very similar to those observed in our samples. According to the observed lesions in cultured sea bass including fin erosion, circular ulcerative lesions and petechial and hemorrhagic skin in abdominal and thoracic regions we ascribe them to vibriosis-like disease (Toranzo *et al.*, 2005).

We have performed a classical bacteriological characterization in water and fish tissue samples. The analysis of internal tissues revealed very similar

microbiota in apparently healthy and diseased fish. Its characterization by API20E led us to identify *V. alginolyticus*, *A. hydrophila*, *Pasteurella multocida*, *Proteus vulgaris* and *P. mirabilis*. This makes difficult to ascertain the real etiologic agent producing the disease and is an important handicap in the field studies. *V. alginolyticus* constitutes an opportunistic invader of already damaged tissues, or a weak pathogen of stressed fish, and has been isolated from water and/or sea bass tissues (Damir *et al.*, 2013; Sadok *et al.*, 2013) as in our study. *A. hydrophila* also produces skin ulcers and hemorrhagic septicemia but is rare in marine waters (Austin and Austin, 2007). However, it has been isolated as the single bacterium from skin ulcers and hemorrhages in marine fish such as Atlantic salmon (*Salmo salar*), European sea bass and sharpnose seabream (*Puntazzo puntazzo*) (Candan and Küçüker, 1995; Doukas *et al.*, 1998). However, the other identified bacteria are not usual in marine fish tissues or related to fish pathology. *P. vulgaris* and *P. mirabilis*, though mainly pathogenic for humans, have been also isolated from fresh and marine water fish (Drzewiecka, 2016) including from fish skin ulcers (Mandal *et al.*, 2002) though their pathogenic properties to fish have not been clearly demonstrated. Interestingly, to the best of our knowledge, *P. multocida* has not been previously identified in fish. As pointed in the literature, the classical identification of marine microorganisms is sometimes difficult and nowadays the use of molecular-based tools is preferred since they do not only favor the identification of isolated bacteria but they are also useful for the identification of bacteria that do not grow, or are not selected with the culture media used.

Strikingly, the only differentially identified bacterium between healthy and diseased sea bass specimens is *Grimontia hollisae*, formerly included in the *Vibrio* genus. This is a human pathogen producing diarrhea (Hickman *et al.*, 1982) but it has been also isolated from environmental samples. Thus, *G. hollisae* has been recovered from amberjack (*Seriola dumerili*) with vibriosis (Ji *et al.*, 2008) as well as from Nile tilapia (*Oreochromis niloticus*) and marine tilapia (*Oreochromis spilurus*) (Al-Sunaiher *et al.*, 2010). In addition, *G. hollisae* strains have been

shown to be very pathogenic to Nile tilapia (Al-Sunaiher *et al.*, 2010). Most of studies report its lack of growth in TCBS cultures though others document a light growth. In fish, our data together to those from tilapia support that *G. hollisae* from fish grow in TCBS, or at least these strains. Based on this microbiological study of sea bass cultured in heated waters bacterial identification and classification deserves deeper analysis of the isolated and identified bacteria as wells as their potential pathogenicity.

Diseased fish suffer internal/external lesions that may alter, or be a consequence, fish physiology leading to fish death. We have evaluated some physiological markers of tissue damage to ascertain the metabolic and physiological alterations in European sea bass suffering vibriosis-like disease. Thus, we found that most of the analyzed seric metabolic parameters were increased in diseased fish compared to apparently healthy specimens though only the levels of phosphorous reached a significant level indicating a kidney and nutrition alteration. Several parameters failed to reach significance likely due to the great variability among specimens which could be related to the differential time exposed to the bacteria and/or disease history. Thus, these data suggest incipient alteration of kidney, muscle, liver and intestine organs but further studies are needed to clearly demonstrate this hypothesis.

We have also evaluated some aspects of the European sea bass blood innate immunity suffering a vibriosis-like disease at humoral and transcriptomic levels. Very little information exists regarding the fish immunity after natural outbreaks of pathogens and none in the case of bacterial pathogens and European sea bass. Thus, the decreased bactericidal activity suggests that fish could not control the bacterial infection and explain the clear signs of disease compared to the apparently healthy fish, though they show the presence of roughly the same potential pathogens. In the scarce studies on European sea bass, infection with inactivated *P. damselae* subsp. *piscicida* resulted in increased complement, lysozyme and protease activities though failed to change the antiprotease and bactericidal activity (Machado *et al.*, 2015). Thus, the responsiveness of diseased sea bass might explain why they are affected. At gene level, we also found interesting data on sea bass immunity. The expression of T cell marker was increased to a non-significant extent in blood suggesting the lack of adaptive cellular immune response in diseased fish while the marker for B cell did, indicating an adaptive humoral immune response. This could indicate that fish have antigen-producing cells in circulation suggesting fish are mounting an adaptive humoral immune response. Anyway, these antibody levels seem not to be enough to encounter and eliminate the bacteria. By contrast, the marker for eosinophilic granulocytes, *mpx*, resulted significantly down-regulated pointing to a marked eosinopenia. This finding, towards the down-regulated transcription of *c3* and *dic* antimicrobial molecules, could explain the decreased bactericidal activity in serum and increased susceptibility of fish specimens to the opportunistic bacteria leading to the disease and mortalities observed. In laboratory-controlled infections, for example, sub-lethal infection of sea bass with *V. anguillarum* resulted in a general up-regulation of genes related to antimicrobial peptides and

the pro-inflammatory cytokine *illb* genes after less than 1 day but usually unaffected after 72 h of infection in immune tissues such as spleen or head-kidney (Meloni *et al.*, 2015). So, fish immunity seems to decline with the progress and clearance of the infection and could partly explain our results in fish with unknown infection time but always longer the 72 h since the external alterations are quite evident. Further studies are needed to evaluate the sea bass immune response in specimens suffering natural outbreaks and its importance in aquaculture.

**Conclusions:** European sea bass reared in ponds with heated water showed high prevalence of specimens with skin ulcers and lesions resembling a vibriosis-like disease. Microbiological examination resulted in very similar load and diversity of bacteria in tissues from apparently healthy and vibriosis-like diseased fish being *G. hollisae* the only differentially present bacteria in diseased fish specimens. Regarding sea bass metabolism, only the levels of phosphorous were elevated in diseased fish. On the other hand, blood immunity resulted impaired in the transcription of the marker for eosinophils (*mpx*) and antimicrobial peptides (*c3* and *dic*) as well as the total bactericidal activity, which supports the better bacterial growth of opportunistic pathogens and therefore the disease. Further multidisciplinary studies are needed to characterize the fish biology under natural outbreaks and how the intensive fish culture with heated water promotes the appearance and incidence of pathogens.

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**Authors contribution:** DM and MO performed the fish sampling and bacteriological analysis. RC, DM and AC performed metabolic and immune analysis. MO, MAE and AC conceived the study and wrote the paper. All authors critically revised the manuscript and approved the final manuscript.

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